

ORIGINAL ARTICLE

Partial Activation of TrkB Receptors Corrects Interneuronal Calcium Channel Dysfunction and Reduces Epileptogenic Activity in Neocortex following Injury

Feng Gu, Isabel Parada, Tao Yang, Frank M. Longo and David A. Prince

Department of Neurology & Neurological Sciences, Stanford University School of Medicine, Stanford, CA 94305-5122, USA

Address correspondence to David A. Prince, Neurology and Neurological Sciences, Stanford University School of Medicine, Room M016, Stanford, CA 94305-5122, USA. Email: daprince@stanford.edu

Abstract

Decreased GABAergic inhibition due to dysfunction of inhibitory interneurons plays an important role in post-traumatic epileptogenesis. Reduced N-current Ca^{2+} channel function in GABAergic terminals contributes to interneuronal abnormalities and neural circuit hyperexcitability in the partial neocortical isolation (undercut, UC) model of post-traumatic epileptogenesis. Because brain-derived neurotrophic factor (BDNF) supports the development and maintenance of interneurons, we hypothesized that the activation of BDNF tropomyosin kinase B (TrkB) receptors by a small molecule, TrkB partial agonist, PTX BD4-3 (BD), would correct N channel abnormalities and enhance inhibitory synaptic transmission in UC cortex. Immunocytochemistry (ICC) and western blots were used to quantify N- and P/Q-type channels. We recorded evoked (e)IPSCs and responses to N and P/Q channel blockers to determine the effects of BD on channel function. Field potential recordings were used to determine the effects of BD on circuit hyperexcitability. Chronic BD treatment 1) upregulated N and P/Q channel immunoreactivity in GABAergic terminals; 2) increased the effects of N or P/Q channel blockade on evoked inhibitory postsynaptic currents (eIPSCs); 3) increased GABA release probability and the frequency of sIPSCs; and 4) reduced the incidence of epileptiform discharges in UC cortex. The results suggest that chronic TrkB activation is a promising approach for rescuing injury-induced calcium channel abnormalities in inhibitory terminals, thereby improving interneuronal function and suppressing circuit hyperexcitability.

Key words: calcium channel, cortical injury, epileptogenesis, interneuron, TrkB

Introduction

Cortical injury is a major cause of acquired epilepsy, accounting for ~20% of all symptomatic epilepsies (Annegers et al. 1998). Many pathophysiological changes occur following traumatic brain injury (TBI), and among these, decreased GABAergic inhibition due to alterations in inhibitory interneurons is prominent in many animal models and in human brain

(DeFelipe 1999; Cossart et al. 2001; Li and Prince 2002; Kobayashi and Buckmaster 2003; Zhang and Buckmaster 2009; Faria and Prince 2010; Ma and Prince 2012). Evidence for functional abnormalities of interneurons in the partial neocortical isolation (undercut or UC) model of post-traumatic epileptogenesis includes decreased miniature (m)IPSC frequency in pyramidal (Pyr) cells, decreased probability of GABA release (Pr),

increased failures of evoked unitary (u)- and monosynaptic evoked inhibitory postsynaptic currents (eIPSCs), and increased coefficient of variation, all of which indicate abnormal function of presynaptic inhibitory terminals (Li and Prince 2002; Faria and Prince 2010; Ma and Prince 2012). The mechanisms underlying these interneuronal alterations are largely unknown, and therapy to improve the function of surviving interneurons in injured brain is not available. There is an urgent need to develop effective approaches to modify these pathophysiological processes.

Presynaptic terminals release neurotransmitters via a calcium-dependent process, and abnormalities in calcium channels are present in both acute and chronic models of epilepsies. To date, 5 calcium channel subtypes have been identified: L, R, T, N, and P/Q (Tsien et al. 1988, 1995). P/Q and N are the two primary calcium channel subtypes that regulate neurotransmitter release in GABAergic inhibitory terminals of neocortex and hippocampus (Poncer et al. 1997; Reid et al. 2003; Hefft and Jonas 2005; Zaitsev et al. 2007). At inhibitory terminals of fast-spiking (FS) interneurons, P/Q-type channels predominate, whereas N channels are the major subtype expressed in non-FS inhibitory interneurons (Zaitsev et al. 2007; Kruglikov and Rudy 2008). However, P/Q and N channels can coexist in the same terminal (Reid et al. 2003). Dysfunction of N channel is associated with hyperactivity and seizures in mice and drosophila (Beuckmann et al. 2003; Rieckhof et al. 2003). Loss of function mutations of P/Q channel are linked to absence seizures and other types of seizures in humans and rodents (Noebels 2012; Rossignol et al. 2013; Gambardella and Labate 2014). Our previous results showed that the density of N channels is downregulated in GABAergic inhibitory interneurons and the response of eIPSCs to N channel blocker ω -conotoxin is significantly reduced in the UC model of post-traumatic epileptogenesis, suggesting a decreased function of N channels (Faria et al. 2012). N channel dysfunction might contribute to the above-mentioned abnormalities of interneurons and circuit hyperexcitability after cortical injury. Therefore, in order to improve the function of interneurons after injury, one potential approach would be to target the defective N channel and boost its function.

Brain-derived neurotrophic factor (BDNF) is an important trophic factor for development and maintenance of both interneuronal and Pyr cell structure and function (Marty et al. 1997; Rutherford et al. 1997; McAllister et al. 1999; Bolton et al. 2000; Jin et al. 2003; Elmariah et al. 2004). Acting through its tropomyosin kinase B (TrkB) receptor, BDNF potentiates N- and P/Q-type calcium channel signaling and increases GABA release in hippocampal cultures (Baldelli et al. 2002, 2005).

Based on the above in vitro findings, the decreased expression of presynaptic Ca²⁺ channels in interneuronal terminals might be due either to reductions in release of BDNF from Pyr cells onto local inhibitory terminals (Kohara et al. 2007) and/or decreases in TrkB-R expression on parvalbumin (PV) interneurons. Previous results showed that both of these abnormalities are present in UC cortex (Gu et al. 2017). We hypothesized that increased activation of TrkB receptors in vivo might correct the N channel abnormalities in GABAergic presynaptic terminals after cortical injury, improve inhibitory function, and suppress the cortical circuit hyperexcitability in the UC model.

To test this hypothesis, we used a newly designed small molecule, PTX-BD4-3 (BD), developed by Frank Longo and colleagues to selectively activate the TrkB receptor in vivo. BD is a derivative of the TrkB-specific partial agonist parent compound LM22A-4 (Massa et al. 2010) and has higher blood-brain barrier

permeability, longer serum half-life, and better absorption following oral administration compared to the parent compound. Effects on N and P/Q channel expression were assessed using immunocytochemical techniques, western blots, and selective Ca²⁺ channel toxins. We used whole-cell patch clamp recordings of spontaneous (s) and eIPSCs and field potential recordings of epileptiform discharges in in vitro slices from saline- and BD-treated UC rats to assess the functional effects of BD treatment. We show that in vivo BD treatment of UC rats upregulates the density of both N and P/Q channels in GABAergic terminals, enhances the response of monosynaptic eIPSCs to N and P/Q channel blockers, increases GABA Pr, and reduces the incidence of spontaneous and evoked epileptiform discharges in cortical slices from UC rats.

Materials and Methods

All experiments were carried out according to National Institutes of Health Guide for the Care and Use of Laboratory Animals and protocols approved by the Stanford Institutional Animal Care and Use Committee.

Partial Cortical Isolations

Male Sprague–Dawley rats (P21; P0 = date of birth) were deeply anesthetized with ketamine (80 mg/kg i.p.) and xylazine (Rompun 8 mg/kg i.p.), and a ~3 × 5 mm bone window, centered on the coronal suture and extending medially to within 1 mm of the sagittal suture, was removed, leaving the dura intact and exposing a portion of the sensorimotor cortex unilaterally. UCs in the sensorimotor cortex were made as previously described (Hoffman et al. 1994; Graber and Prince 2006). A 30-gauge needle, bent at approximately a right angle 2.5–3 mm from the tip, was inserted parasagittally ~1–2 mm from the interhemispheric sulcus, advanced under direct vision tangentially through the dura and just beneath the pial vessels, and lowered to a depth of 2 mm. The needle was then rotated through 180° to produce a contiguous white matter lesion, elevated to a position just under the pial vessels to make a second transcortical cut, and removed. An additional transcortical lesion was placed ~2 mm lateral and parallel to the initial parasagittal cut in a similar manner. The skull opening was then covered with sterile plastic wrap (Saran Wrap) and the skin sutured. Animals were given carprofen 5 mg/kg s.c. postoperatively. Lesioned animals recovered uneventfully from the surgery.

Protocols for BD Administration

Animals were randomly treated daily with either PTX BD4-3 (50 mg/kg i.p.) or saline once per day for 7 days, beginning within 1 h after surgery. Immunocytochemical and western blot experiments were performed 3 days after the end of treatment and electrophysiological recordings obtained 2–8 days after the last BD dose.

Neocortical Slice Preparation and Field Potential Recordings

Neocortical slice preparation was as previously described (Graber and Prince 2006). After incubation, slices were transferred to an interface chamber where the partially isolated cortical area was easily identified under a dissecting microscope and evoked field potentials recorded in layer V with an artificial

cerebrospinal fluid (ACSF)-filled glass pipette. Focal extracellular 100- μ s single square-wave pulses were delivered at 0.1 Hz through a concentric bipolar electrode (FHC) placed at the layer VI/white matter junction on column with the recording electrode. Stimulus intensity was adjusted to be at threshold for eliciting a short latency field potential response. Recordings were obtained from 2 to 3 slices cut through the UC in each rat and through the comparable cortical area from controls. Stimulating and recording electrode pairs were moved together across the slice to up to 5 sites to be certain that “hot spots” for evoking epileptiform responses were not missed in slices when epileptiform responses were not initially evoked (Graber and Prince 2004). Stimuli in epileptogenic slices elicited interictal epileptiform events that were readily distinguished from other responses based on their variable and often long latency and duration, their occurrence at a threshold as all-or-none responses, and their polyphasic contours with peaks often associated with bursts of extracellular action potentials (Prince and Tseng 1993; Hoffman et al. 1994). Similar epileptiform events occurred spontaneously in some slices.

Whole-Cell Patch Clamp Recordings

Male rats were anesthetized, and using previously described techniques (Faria and Prince 2010), brains were removed and coronal neocortical slices cut and maintained for in vitro slice recordings. Neocortical slices (~300 μ m) were cut with a vibratome in cold (4 ± 1 °C) “slicing” ACSF containing (in mM): 126 NaCl, 2.5 KCl, 1.25 NaH_2PO_4 , 1 CaCl_2 , 2 MgSO_4 , 26 NaHCO_3 , and 10 glucose; pH 7.4, when saturated with 95% O_2 –5% CO_2 . After 1 h of incubation in standard ACSF containing (in mM) 126 NaCl, 2.5 KCl, 1.25 NaH_2PO_4 , 2 CaCl_2 , 1 MgSO_4 , 26 NaHCO_3 , and 10 glucose (32 ± 1 °C), single slices were transferred to a recording chamber where they were minimally submerged (32 ± 1 °C) and perfused at the rate of 2.5–3 mL/min with standard ACSF. Patch electrodes were pulled from borosilicate glass tubing (1.5 mm OD) and had impedances of 4–6 $\text{M}\Omega$ when filled with intracellular solution containing (in mM): 60 K-gluconate, 67 KCl, 0.1 CaCl_2 , 10 HEPES, 1.1 EGTA, 4 ATP-Mg, and 6 phosphocreatin-tris and 0.2% biocytin. The osmolarity of the pipette solution was adjusted to 285–295 mOsm and the pH to 7.35–7.4 with KOH.

Whole-cell voltage clamp recordings were made from visually identified layer Va Pyr cells using infrared video microscopy and differential interference contrast optics (Zeiss Axioskop2) and a Multiclamp 700A amplifier (Axon Instruments). The estimated chloride equilibrium potential (E_{Cl}) was -18 mV based on the Nernst equation. Access resistance (R_a) was measured in voltage clamp mode from responses to 2 mV hyperpolarizing voltage pulses. Data from recordings in which R_a varied by more than 15% or was >20 $\text{M}\Omega$ were rejected. Pyramidal neurons were identified as cells with large somata and a single emerging apical dendrite extending toward the pial surface. In addition, in some slices, intracellular labeling with biocytin was used to confirm cell type and position. Pharmacologically isolated (monosynaptic) IPSCs were reliably evoked with monopolar tungsten-stimulating electrodes, placed in layer V, ~80 μ m below the recorded Pyr soma to obtain steep input/output slopes (Salin and Prince 1996). 2-Amino-5-phosphonovaleric acid (D-AP5; 50 μ M) and 6,7-dinitroquinoxaline-2,3-dione (DNQX; 20 μ M) (Ascent Scientific) in ACSF were continuously applied via bath perfusion. ω -Conotoxin GVIA (1 μ M, Ascent Scientific) and ω -agatoxin IVa (0.2 μ M, Bachem Bioscience) were bath applied to

assess the effects of N channel and P/Q channel blockade on eIPSCs, respectively.

To obtain the threshold (T) for evoking IPSCs, the stimulus duration was initially set at 100 μ s and stimulus current increased until a stable evoked (e)IPSC with a failure rate of $\leq 50\%$ was evoked. The pulse duration was then increased to 150 μ s (1.5 T) for determination of peak amplitude. In some experiments, bath perfusion of gabazine (10 μ M) was used to block the evoked events and verify that they were mediated by GABA_A receptors (not shown). Responses in which spontaneous (s) IPSCs were superimposed on evoked IPSCs were not included in the data analysis.

Baseline values for eIPSC peak amplitude were obtained in UC rats during the first 5 min of stable whole-cell recording in standard ACSF. This was followed by a single 10-min local perfusion of ω -agatoxin IVa and/or ω -conotoxin GVIA. The effects of calcium channel blockade on eIPSCs were routinely assessed for 5 min, beginning after the end of toxin perfusion. Mean values for peak amplitude were calculated by averaging eIPSCs over 5 min prior to, and after the 10-min toxin treatment in UC. The prolonged actions of the toxins did not allow washout of the effects. Statistical significance was determined with two-tailed Student's t-test ($P < 0.05$). Data are expressed as mean \pm SEM and “n” corresponds to the number of neurons, unless otherwise noted. One neuron was recorded per slice and no more than 3 slices were used per rat. “Baseline” values below are those for UCs before toxin exposure.

Immunocytochemistry

P31 male UC rats treated with BD or saline were deeply anesthetized with Beuthanasia-D (110 mg/kg) and perfused transcardially with 4% paraformaldehyde. Coronal brain sections (40 μ m) were cut with a microtome (Microm, HM 400). Free floating sections were incubated for 1 h in 10% normal goat serum in phosphate-buffered saline (PBS), followed by 48-h incubation with the primary antibodies against N channel (1: 200), P/Q channel (1: 200) (Alomone Labs, Israel and Sy Sy Germany, respectively), or cholecystokinin (CCK, 1: 500, CURE) in combination with an antibody for vesicular GABA transporter (VGAT) (Sy Sy Germany; 1: 500) or PV (1, 1000, Sigma) diluted in PBS containing 0.3% (v/v) Triton X-100. Subsequently, slices were rinsed in PBS and incubated for 2 h with the corresponding fluorescent secondary antibodies (Jackson Immuno Research Laboratories). Sections were mounted on slides using Vectashield Mounting Media. Images were captured with a laser scanning confocal microscope (Zeiss LSM 880) by using $\times 40$ and $\times 63$ oil immersion lens and a zoom factor of 2. Secondary antibodies tagged to Fluorescein 488 and Cy3 were excited with 488- and 543-nm lasers and observed through 510–530 and 560–615 emission filters, respectively. A pinhole of 1 airy unit and identical settings for detector gain and amplifier offset were used to capture all confocal images.

Quantification of immunoreactivity (IR) for the antibodies against N or P/Q channel+ VGAT was performed in terminals projecting onto the somata of layer Va Pyr cells. For volumetric analysis of perisomatic objects, Pyr cells were first identified in confocal image stacks as neurons with triangular-shaped somata and an apical dendrite projecting toward the pial surface. The image stack was scanned and cropped in the z-dimension to encompass 4–5 sequential 0.37 μ m optical sections through the approximate center of the Pyr cell soma. VGAT-IR was used as a guide to demarcate the perisomatic region, as it associates closely with the somata of Pyr neurons (e.g., Fig. 1)

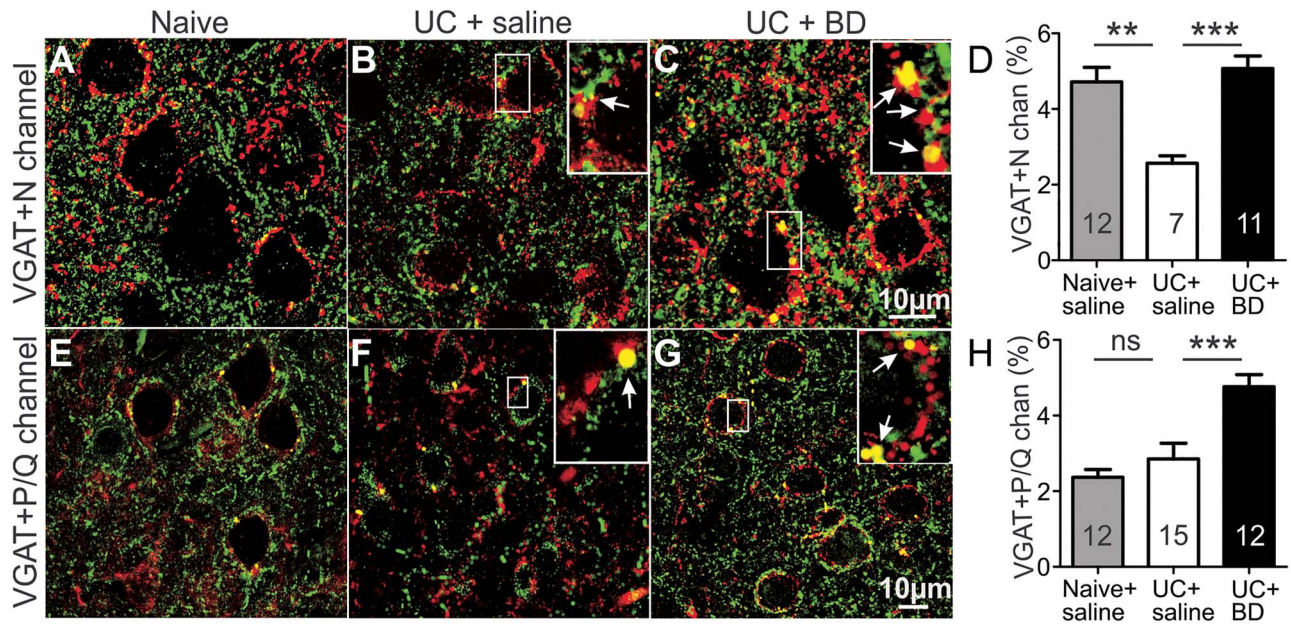


Figure 1. Increased perisomatic density of N- and P/Q-type calcium channel IR in BD-treated UC inhibitory terminals. Confocal images of dual IR for VGAT (red) + N channel (green) from layer Va sections from naïve (A), and UC rats treated with saline (B) and BD (C). Small white boxes in B, C, F, and G mark representative puncta with dual labeling (yellow) touching cell somata, seen enlarged in large white box at the upper right of each frame. Arrows in large boxes point to perisomatic sites of colocalization of VGAT and N channel-IR (yellow). VGAT- (red) + P/Q channel-IR (green) in layer Va of slices from naïve (E), saline-treated (F), and BD-treated UC rats (G). (C, G) Confocal images show increased VGAT-IR in BD-treated UC rats versus images from saline-treated UC rats in B, F. Perisomatic colocalization of VGAT/N channel-IR (C) and VGAT/P/Q channel-IR (G) is increased by BD treatment in UC rats. (D, H) Bar graphs showing mean percentage of perisomatic colocalization of VGAT/N channel-IR (D) and VGAT/P/Q channel-IR (H) for Pyr cells in layer Va. Results are expressed as total IR volume/volume of perisomatic ROI and normalized. Numbers in bars: number of cells. ** $P < 0.01$; *** $P < 0.001$ by post hoc Tukey test following one-way ANOVA.

(Chaudhry et al. 1998). The inner boundary of the region of interest (ROI) was drawn at the junction of positive perisomatic immunostaining for VGAT and the void representing the cell body. The outer boundary was drawn to include immunoreactive VGAT puncta in close apposition to the soma. This resulted in a $\sim 1.5\text{-}\mu\text{m}$ wide “train track” around the perisomatic region that extended $\sim 1.8\text{ }\mu\text{m}$ in the z-dimension. Objects within the ROI for a given channel (e.g., Cy-3-tagged perisomatic proteins) were identified using Imaris software (Bitplane) if their pixel intensities were greater than 2 SD away from the mean pixel intensity and the voxel size was $>0.2\text{ }\mu\text{m}^3$. The cumulative volume of the identified objects (i.e., the cumulative volume of the perisomatic colocalized IR for P/Q- or N-type channels with the presynaptic marker VGAT or PV) was compared to the total volume of the perisomatic ROI of layer V Pyr cells. The results showed the percentage of cumulative colocalized voxels/total volume of perisomatic ROI.

Western Blots

Fresh full-thickness cortical brain tissue was dissected from saline-treated UC and BD-treated UC cortical areas and sonicated in homogenization buffer: 12.5% 0.5 M Tris-HCl, 10% glycerol, 2% SDS and protease inhibitor cocktail (Thermo Scientific). Homogenates were centrifuged at 14000g for 15 min at 4 °C and supernatant collected. Samples of protein (15–20 μg) were separated by 4–15% Tris-HCl running gel and transferred to Amersham Hybond-P transfer membranes (GE Healthcare). Membranes were blocked with 5% nonfat milk in TBST buffer for 1 h at room temperature and incubated overnight at 4 °C with primary antibodies against N-type calcium channel (Sy

Sy Germany; 1:1000) and P/Q-type calcium channel (Sy Sy Germany; 1:1000). After several washes, the membranes were incubated with secondary antibodies at dilutions of 1: 20000 for 1.5 h at room temperature. After further extensive washing, the immunoreactive bands were detected with ECL plus western blot detection system reagents (GE healthcare). Quantification of optical density (OD) of western blots was performed using Un-Scan-It gel software (v6.1, Silk Scientific). Relative expression of specific protein was normalized and calculated as the OD of specific protein/OD of β -actin.

Statistical Analyses

Data are expressed as mean \pm SEM and were analyzed using GraphPad Prism software. Statistical significance of differences ($P < 0.05$) was measured using two-tailed Student t-tests, one-way analysis of variance (ANOVA), or Fisher's exact test. Analyses of immunocytochemical and electrophysiological data were done by blinded observers.

Results

Effect of Chronic in vivo BD Treatment on the Density of N and P/Q Channels in GABAergic Terminals following Cortical Injury

Previous results had shown a reduction of N channels in perisomatic GABAergic terminals in UC versus control Pyr neurons (Faria et al. 2012). Here, ICC was used to compare the effects of in vivo BD treatment versus saline treatment on the expression of N and P/Q channels in GABAergic terminals after UC cortical injury. We used layer Va Pyr cell perisomatic VGAT IR to image

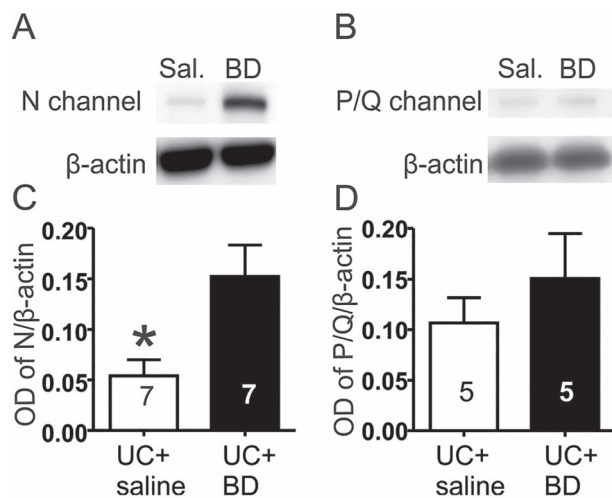


Figure 2. Chronic BD treatment increases protein level of N channel in UC cortex. (A, B) Representative immunoblots of N channel (A) and P/Q channel (B) from saline-treated (Sal.) and BD-treated UC rats. (C, D) OD of N and P/Q immunoblots normalized to the expression of β actin. (C) Graph shows that BD treatment upregulates N channel protein level in UC cortex. (D) There is no significant increase in P/Q channel expression ($P=0.41$). Numbers in bars: number of rats. * $P < 0.05$ by unpaired t-test. Data are expressed as mean \pm SEM.

GABAergic terminals from interneurons and dual IR for VGAT and N or P/Q channels to identify the two major calcium channels that regulate neurotransmitter release in inhibitory terminals (Fig. 1). Our results showed that the volume of colocalized VGAT- and N channel-IR in the perisomatic ROI in BD-treated UC group ($5.07 \pm 0.33\%$, $N=11$) was significantly greater than in the saline-treated UC group ($2.57 \pm 0.2\%$, $N=7$, post hoc Tukey test following one-way ANOVA; $P < 0.001$) (Fig. 1B–D). The colocalized VGAT- and P/Q channel-IR in perisomatic ROI was also increased in the BD-treated ($4.76 \pm 0.32\%$, $n=12$) versus saline-treated group ($2.85 \pm 0.41\%$, $n=15$, post hoc Tukey test following one-way ANOVA; $P < 0.001$) (Fig. 1F–H). These ICC results indicate that chronic BD treatment can upregulate the expression of both N and P/Q channels in GABAergic terminals following neocortical injury.

Effect of BD Treatment on N and P/Q Channel Proteins in UC Cortex

To further examine the effect of chronic BD treatment on the expression of N and P/Q channels in the UC model, western blot was performed. The results showed that, when normalized to β -actin, BD treatment significantly increased N channel protein level in the UC rats (optical density: UC + BD 0.15 ± 0.03 , $n=7$ rats, vs. UC + saline 0.05 ± 0.02 , $n=7$ rats, $P=0.003$) (Fig. 2A,C). There is no significant increase in P/Q channel protein level in BD-treated UC rats. (UC + BD 0.15 ± 0.04 , $n=5$ rats vs. UC + saline 0.11 ± 0.03 , $n=5$ rats, $P=0.41$; Fig. 2B,D). Consistent with the ICC results, western blot analysis confirmed the significant increase in N channel expression in UC rats after BD treatment compared to saline-treated UC rats. The discrepancy in the results for P/Q channel between ICC and western blot could be due to the sensitivity of these two different techniques, antibody used, and the fact that western blot quantified the P/Q protein level in the sample that contains all six layers of the cortex while ICC selectively targeted only layer Va.

Effects of Chronic in vivo BD Treatment on the Function of N and P/Q Channels and GABAergic Synaptic Transmission

To determine whether the BD-induced alterations in the expression of N and P/Q channels found in ICC and western blot experiments were associated with changes in inhibitory function, we next examined the effects of blocking these channels on monosynaptic eIPSCs in layer Va Pyr cells of UC slices. If in vivo BD treatment increases eIPSCs through effects to increase expression of N and/or P/Q channels (Figs 1 and 2), snail toxins selective for these channels should decrease eIPSCs to a greater extent in BD- versus saline-treated UC rats in in vitro slice experiments. Results showed that there was a larger reduction in eIPSC amplitude in BD-treated UC rats than in saline-treated UC rats after N channel blockade with ω -conotoxin (Fig. 3A,B; eIPSC amplitude: 73 ± 13.7 pA in UC + BD group ($n=7$) vs. 24.7 ± 7.8 pA in UC + saline controls ($n=5$); unpaired t-test, $P=0.021$). P/Q channel blockade with agatoxin also induced a larger decrease in eIPSC amplitude in BD-treated UC versus saline-treated UC rats (Fig. 3D,E; UC + BD 161.7 ± 31.2 pA, $n=7$; UC + saline 73.5 ± 15.4 pA, $n=5$; unpaired t-test, $P=0.05$). These results show that chronic BD treatment can enhance the function of both N and P/Q channels in the terminals of interneurons of UC cortex.

Because N and P/Q channels are important for regulating neurotransmitter release from inhibitory terminals, we next assessed the paired pulse ratio (PPR) for eIPSCs as an index of release probability (Pr). After blockade of glutamatergic transmission with DNQX ($20 \mu\text{M}$) and APV ($50 \mu\text{M}$), eIPSCs were evoked on layer V Pyr cells in UC slices with pairs of $1.5 \times$ threshold stimuli (50 ms interstimulus interval) delivered by an electrode positioned within $\sim 80 \mu\text{m}$ of the cell soma. The mean PPR ($R2/R1$), calculated after excluding response failures, was significantly smaller in 18 neurons from BD-treated UC rats than in 13 neurons from saline-treated UC rats (PPR 0.72 ± 0.03 vs. 1.00 ± 0.06 , unpaired t-test, $P < 0.05$) (Fig. 3I), suggesting that BD treatment increased GABA Pr. Increases in eIPSC amplitudes (Fig. 3G vs. Fig. 3H) were also present in slices from BD- vs. saline-treated UC rats, confirming results obtained with LM22A-4, another small molecule selective TrkB partial agonist (Gu et al. 2018).

To determine whether the increases in Pr induced by BD treatment would affect ongoing inhibitory synaptic activity, we recorded spontaneous (s)IPSCs from layer Va Pyr cells in saline- and BD-treated UC slices (Fig. 4A). The frequency of sIPSCs in Pyr cells from BD-treated UC rats was significantly increased (8.02 ± 1.31 Hz; $n=6$ rats) versus saline-treated UC animals (4.30 ± 0.56 Hz; $n=6$ rats) (unpaired t-test, $P < 0.05$) (Fig. 4B) with no significant difference in sIPSC amplitude (Fig. 4C). We ruled out effects of BD on resting membrane potential; input resistance; and amplitude, threshold, and frequency of action potentials of FS interneurons in layer Va that might have accounted for increases in spontaneous IPSC frequency (Fig. 4D–I). In the absence of effects of TrkB activation to increase excitatory transmission and epileptogenesis in the UC model (Gu et al. 2018 and Fig. 5 in current results), the results suggest that the increased GABA Pr is one factor contributing to the increased sIPSC frequency induced by BD treatment.

Effect of BD Treatment on Excitability in Neocortical Networks

Spontaneous behavioral seizures occur in animals with partial neocortical isolations (Graber and Prince 2006; Chauvette et al.

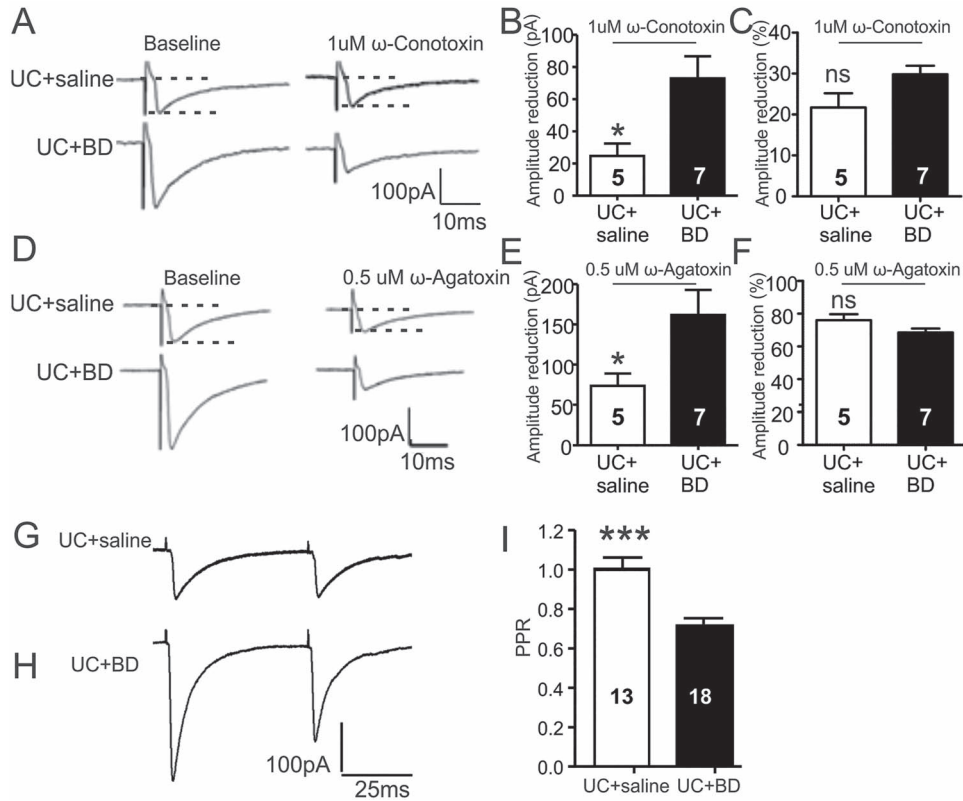


Figure 3. BD treatment increases response of monosynaptic eIPSC to both N channel and P/Q channel blockades. (A, D) Representative traces of single eIPSCs evoked in layer Va Pyr neurons from rat neocortical slices from saline-treated UC (upper traces) and BD-treated UC (lower traces). Baseline traces (left column): Responses in ACSF. Right traces: Responses in same slices after perfusion with ACSF containing 1 μ M ω -conotoxin GVIA (A) or 0.5 μ M ω -agatoxin IVa (D). Dashed lines mark onsets and peaks of UC + saline traces in A, D. (B, E) Change in the peak amplitude of eIPSCs in BD-treated UC (black bar) is greater than in saline-treated UC (white bar) after either N channel (B) or P/Q channel blockade (E). (C, F) Percentage change in amplitude of eIPSCs in BD-treated UC and saline-treated UC after either N channel (C) or P/Q channel blockade (F). (G, H) Representative responses to 1.5 \times threshold paired stimuli (ISI=50 ms) from saline-treated (E) and BD-treated (F) UC neurons. (I) Mean PPR for eIPSCs in BD-treated UC neurons is significantly decreased compared with that in saline-treated UC neurons. Numbers in bars: number of cells. * $P < 0.05$; *** $P < 0.001$ by unpaired t-test. All stimuli delivered at 1.5 \times threshold.

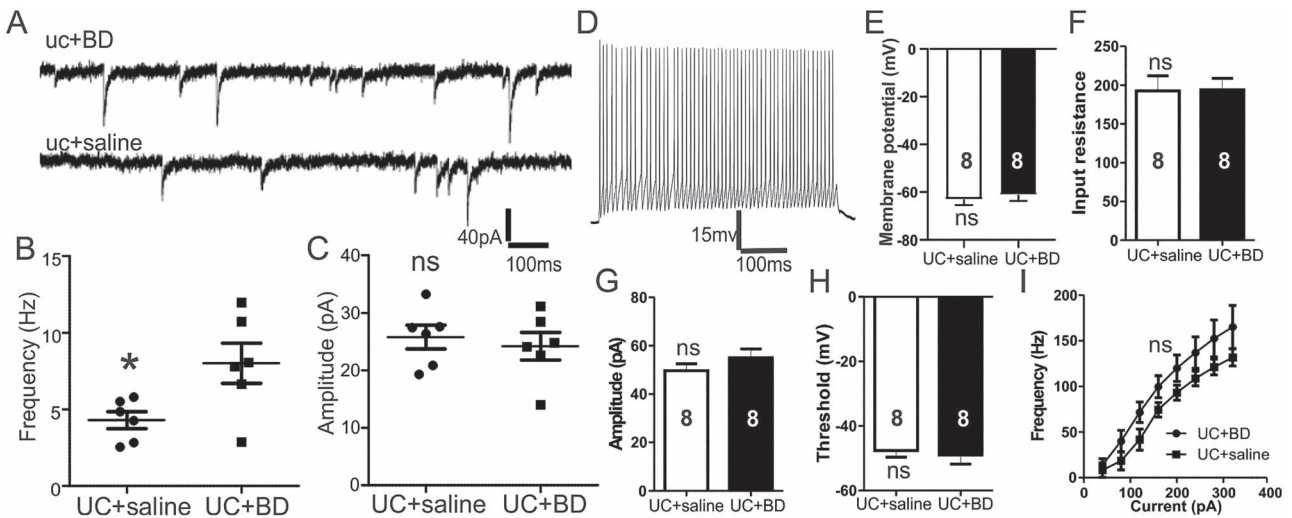


Figure 4. Chronic BD treatment of UC rats increases inhibitory synaptic transmission. (A) Representative traces of sIPSCs recorded from layer V Pyr cells in saline-treated UC (lower trace) and BD-treated UC cortex (upper trace). Graphs show a significant increase in sIPSC frequency (B), without a significant change in amplitude (C). (D) Representative trace of directly evoked action potential firing in a FS PV interneuron. (E-I) No significant differences in resting membrane potential (V_m); input resistance; amplitude, threshold, and frequency of action potentials of FS interneurons in UC rats treated with BD (black bar) versus UC saline controls (white bar). Numbers in bars: number of cells. * $P < 0.05$ by unpaired t-test.

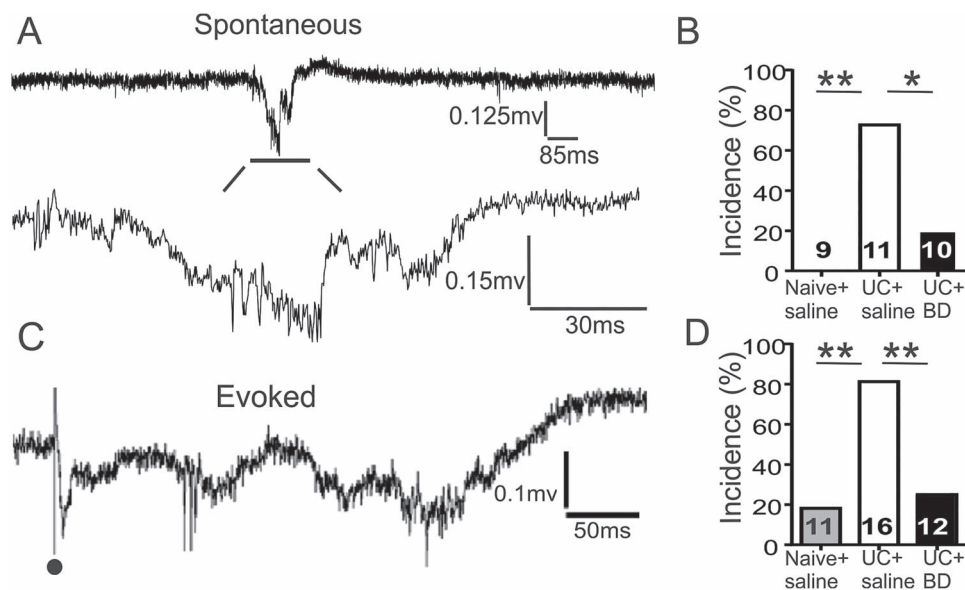


Figure 5. Chronic BD treatment suppresses epileptiform discharges in UC slices. Representative traces of spontaneous (A) and evoked (C) epileptiform discharges (black dot in C marks stimulus artifact). Lower trace in (A): Selected segment from the upper trace at higher gain and expanded time base. Bar graphs show the incidence of spontaneous (B) and evoked (D) epileptiform discharges in naïve + saline (gray, left), UC + saline (white), and UC + BD (black) groups. No spontaneous bursts were recorded in slices from naïve rats. In vivo BD treatment significantly decreased the incidence of both spontaneous and evoked epileptiform discharges in UC slices. Numbers in bars of C, D: numbers of slices (2–3 slices/rat). * $P < 0.05$; ** $P < 0.01$ by Fisher's exact test.

2016; Ping and Jin 2016), and UC slices are hyperexcitable and generate spontaneous and evoked epileptiform bursts (Hoffman et al. 1994; Graber and Prince 1999; Li et al. 2012). Two critical factors underlying these abnormalities are axonal sprouting associated with excessive excitatory synaptogenesis (Salin et al. 1995) (Li and Prince 2002) and decreases in GABAergic transmission due to presynaptic abnormalities in interneurons (Ma and Prince 2012; Gu et al. 2017, 2018). We hypothesized that, by enhancing GABAergic transmission slices from UC cortex, BD treatment would reduce the capacity of the aberrant excitatory networks to generate epileptogenic events. Extracellular field potential recordings from neocortical layer V showed that spontaneous epileptiform bursts occurred in 72.7% of slices from UC saline-treated rats (Fig. 5B, 8/11 slices, 2–3 slices/rat) but were not present in naïve slices (Fig. 5B). In vivo BD treatment resulted in a significant reduction in incidence of spontaneous bursts in slices from UC rats (Fig. 5B; 20%, 2/10 slices) (Fisher's exact test, $P < 0.05$). There was a low incidence of evoked bursts in naïve slices from saline-treated animals (18.2%, 2/11 slices; Fig. 5D). The incidence was increased significantly in slices from saline-treated UC rats (81.3%, 12/16 slices; Fig. 5D) ($P < 0.01$ Fisher's exact test). The percentage of UC slices with epileptiform bursts was similar to that previously reported (Hoffman et al. 1994). In vivo BD treatment markedly decreased the capacity of slices from UC rats to generate evoked epileptiform bursts and brought the incidence of evoked bursts down to 25% (3/12 slices) (Fisher's exact test, $P < 0.01$) (Fig. 5D). Taken together, these findings show that chronic in vivo BD treatment following neocortical injury can upregulate N and P/Q channels, increase GABAergic inhibition, and suppress epileptogenesis, measured

as the incidence of spontaneous and evoked epileptiform burst activity in in vitro slices.

Discussion

Dysfunction of interneurons and resulting decreased GABAergic inhibition contributes to epileptogenesis in animal models and man (refs above). Interneuronal terminals release GABA via a calcium-dependent process, and abnormalities in calcium channels are present in both acute and chronic models of epilepsies (Faria et al. 2012; Rossignol et al. 2013; Bomben et al. 2016). P/Q and N are the two primary calcium channel subtypes that regulate neurotransmitter release from GABAergic inhibitory terminals of neocortex and hippocampus (Poncer et al. 1997; Reid et al. 2003; Hefft and Jonas 2005; Zaitsev et al. 2007). Their dysfunction has been implicated in hyperactivity and seizures (Beuckmann et al. 2003; Rieckhof et al. 2003; Noebels 2012). Previous results in the UC model of post-traumatic epilepsy (PTE) have shown abnormalities in presynaptic terminals of PV interneurons (Ma and Prince 2012), including a decreased expression of N-type channels, associated with decreased GABA release (Faria et al. 2012) that may contribute to hyperexcitability following cortical injury. This suggests that increasing N and/or P/Q channel function may be an effective approach to increase GABAergic inhibition and decrease PTE.

BDNF can have long-lasting effects to increase the frequency of GABA release in immature cultured hypothalamic (Obrietan et al. 2002) and hippocampal neurons (Baldelli et al. 2005). Its trophic actions in hippocampal cultures primarily affect presynaptic terminal function, by increasing Pr and synthesis of N- and P/Q-type calcium channels (Baldelli et al. 2000, 2002, 2005). These results, and the availability of a small molecule TrkB partial agonist, led us to test the effects of chronic TrkB activation on N- and P/Q channel expression in presynaptic GABAergic terminals in the UC model. The reduction in amplitude of monosynaptic (e)IPSCs in layer V Pyr cells in UC slices

following bath perfusion of the specific N channel blocker ω -conotoxin or P/Q channel blocker agatoxin was measured as an index for N and P/Q channel function (Faria et al. 2012). In addition, immunocytochemical experiments were performed to assess the density of N and P/Q channel in presynaptic GABAergic terminals by measuring the colocalization of VGAT-IR with N- or P/Q-IR. Our results in injured mature neocortex show that chronic BD treatment after injury increases responses of monosynaptic eIPSCs to both N channel and P/Q channel blockade (Fig. 3) and increases colocalization of VGAT-IR with N- and P/Q-IR in the perisomatic region of Pyr cells (Fig. 1). These findings are consistent with previous reports that BDNF upregulates P/Q and N channel expression and potentiates their signaling in inhibitory terminals in embryonic hippocampal cultures via TrkB activation (Baldelli et al. 2000, 2002, 2005). However, although above findings suggest that BD treatment increases density of P/Q and/or N channel in perisomatic inhibitory VGAT-containing terminals, we cannot rule out the possibilities that increased perisomatic colocalization of VGAT- and P/Q or N channel-IR may arise from increased number or size of VGAT puncta, and/or higher percentage of VGAT puncta that are P/Q and N channel positive. Array tomography or quantitative EM might be an approach to directly investigate this issue in future experiments.

The interneuronal subtype that showed upregulated N- and P/Q-IR in perisomatic GABAergic terminals after BD treatment (Fig. 1) might be either PV or CCK basket cells that target Pyr cell somata in neocortex and hippocampus (Pawelzik et al. 2002; Karson et al. 2009; Lee et al. 2011; Faria et al. 2012). P/Q and N channels predominate in terminals of PV and CCK cells, respectively (Poncer et al. 1997; Hefft and Jonas 2005; Zaitsev et al. 2007). Our ICC results also showed prominent perisomatic VGAT- and PV-IR colocalized with both P/Q- and N channel-IR in layer Va of neocortex (Fig. 1 and Supplementary Fig. 1), suggesting that P/Q and N channels both exist in PV terminals (Supplementary Fig. 1, see also Reid et al. 2003). As expected, further analysis of our ICC results shows that colocalized PV + P/Q channel-IR and PV + N channel-IR are both increased by BD treatment in the perisomatic ROI of layer Va Pyr cells (Supplementary Fig. 1), consistent with increased perisomatic colocalization of VGAT+P/Q channel-IR and VGAT+N channel-IR (Fig. 1). In neocortical layer V, there are abundant PV interneurons, while CCK cells are rare (Uematsu et al. 2008), suggesting that BD upregulation of perisomatic P/Q and N channels in layer Va of injured neocortex may arise mainly from the effects of BD treatment on PV interneurons (Gu et al. 2018), with less contribution from CCK interneurons. This is further supported by additional ICC results showing unchanged perisomatic colocalized VGAT/CCK-IR in neocortical layer Va (Supplementary Fig. 2). Two-photon confocal microscopic imaging will be helpful to further assess the effect of BD on distribution and density of N and P/Q channels in presynaptic terminals of PV and CCK interneurons.

Increases in perisomatic P/Q and N channels are consistent with electrophysiological results, showing that BD treatment increased Pr (decreased the PPR) for eIPSCs in layer Va Pyr cells of UC slices (Fig. 3). The effects to increase Pr and sIPSC frequency (Figs 3 and 4) were similar to those previously reported in experiments with a different TrkB partial agonist, LM22A-4, in the UC model (Gu et al. 2018). The increased frequency of sIPSCs (Fig. 4) likely results mainly through actions on PV interneurons that provide the predominant GABAergic input to Pyr cell somata, as discussed above. Additional experiments will be required to determine the relative contributions to the BD

effects by PV and CCK interneurons, the two major inhibitory cell types presynaptic to Pyr cell somata.

Loss of perisomatic N channels may be implicated in the dysfunction of PV interneurons (Faria et al. 2012) that contributes to decreased GABAergic inhibition and epileptogenesis after cortical injury (Ma and Prince 2012; Gu et al. 2017). The upregulation of perisomatic N and P/Q channels and GABAergic synaptic transmission by BD led us to test whether BD treatment can suppress epileptogenesis in UC model, in which spontaneous and evoked epileptiform burst discharges occur in the injured cortex. Field potential recordings from UC cortical slices showed that the incidence of spontaneous or electrically evoked "interictal" epileptiform bursts was significantly decreased in the BD-treated group (Fig. 5), suggesting that chronic partial TrkB activation by BD has antiepileptogenic effects, consistent with recent results showing that BD treatment decreases the susceptibility to PTZ-induced seizures in UC rats (Gu et al. 2018).

It should be noted that, apart from its above effect on GABAergic inhibition, TrkB activation may also enhance sprouting or transmitter release from excitatory axons and increase excitatory synaptic transmission (Carmignoto et al. 1997; Bolton et al. 2000). Effects of BDNF activation of TrkB receptors in animal models of epilepsy are controversial, with reports of both increases (Kokaia et al. 1995; McKinney et al. 1997; Croll et al. 1999; Zhu and Roper 2001; Gill et al. 2013; Liu et al. 2013; Gu et al. 2015; Kang et al. 2015) and decreases (Reibel et al. 2003; Paradiso et al. 2009; Cai et al. 2016; Eftekhari et al. 2016; Prince et al. 2016; Gu et al. 2018) in epileptogenesis. Such apparently opposite results could be due to temporal factors, for example, acute application of BDNF can reduce synaptic inhibition (Zhu and Roper 2001) while chronic treatment increases inhibition (Yamada et al. 2002). Also, there are opposing effects of BDNF at its TrkB and p75 receptors (Chapleau and Pozzo-Miller 2012); and potential differences in concentration and distribution of exogenous versus activity-induced, endogenously released BDNF. Differences in the downstream cascades activated, and in particular the experimental models used in studies of TrkB-R activation, may also contribute to discrepant results (Gu et al. 2018). BD, the small molecule used in the present study, has better blood-brain barrier penetration than LM22A-4 used in our earlier experiments (Gu et al. 2018) (Yang et al., unpublished results). Unlike BDNF, these small molecules only partially activate the TrkB receptor and its downstream pathways, and also do not bind to or modulate the p75 receptor (Massa et al. 2010), and thereby may have different effects on epileptogenesis. It will be important in the future to examine the contributions of each TrkB-activated pathway to pro- and antiepileptogenic effects.

In summary, our data suggest that chronic TrkB activation might be a promising strategy to rescue the injury-induced N channel abnormalities and enhance P/Q channel function in GABAergic terminals and thereby increase GABAergic inhibitory transmission and suppress post-traumatic epileptogenesis.

Supplementary Material

Supplementary material can be found at CERCOR online.

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Conflict of Interest

F.M.L. is listed as an inventor on patents relating to LM22A-4, which are assigned to UNC and UCSF, and is eligible for royalties distributed by the assigned universities. He has financial interest in Pharmatrophix, a company focused on the development of small molecule ligands for neurotrophin receptors, which has licensed several of these patents. He is also listed as an inventor on a patent related to PTX BD4-3, which is assigned to Pharmatrophix.

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